

# Differential expression of mRNA in human thyroid cells depleted of mitochondrial DNA by ethidium bromide treatment

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## ABSTRACT

A wide variety of human diseases have been associated with defects in mitochondrial DNA (mtDNA). The exact mechanism by which specific mtDNA mutations cause disease is unknown and, although the disparate phenotypes might be explained on the basis of impaired mitochondrial gene function alone, the role of altered nuclear gene expression must also be considered. In recent years, the experimental technique of depleting cells of mtDNA by culturing them with ethidium bromide has become a popular method of studying mitochondrial disorders. However, apart from depleting mtDNA, ethidium bromide may have many other intracellular and nuclear effects. The aim of the present study was to investigate the effects of ethidium bromide treatment on nuclear gene expression. A simian-virus-40-transformed human thyroid cell line was depleted of mtDNA by culture in ethidium bromide, and differential display reverse transcriptase-PCR (DDRT-PCR) was then employed to compare mRNA expression between wild-type, mtDNA-replete ( $\rho^+$ ) and ethidium bromide-treated, mtDNA-depleted ( $\rho^0$ ) cells. Expression of the majority of nuclear-encoded genes, including those for subunits involved in oxidative phosphorylation, remained unaffected by the treatment. Seven clones were found to be underexpressed; three of the clones showed significant similarity with sequences of the human genes encoding RNase L inhibitor, human tissue factor and ARCN1 (archain vesicle transport protein 1), a highly conserved species which is related to vesicle structure and trafficking proteins. We conclude that the effects of ethidium bromide treatment on nuclear gene expression are not simply limited to changes in pathways directly associated with known mitochondrial function. Further studies will be required to elucidate which of these changes are due to mtDNA depletion, ATP deficiency or other disparate effects of ethidium bromide exposure. Given that most genes appear unaffected, the results suggest that depleting cells of mtDNA by ethidium bromide treatment is a valuable approach for the study of mitochondrial mutations by cybrid techniques.

## INTRODUCTION

Defects in mitochondrial DNA (mtDNA) have been implicated in a variety of human diseases, including

Leber's hereditary optic neuropathy, Kearns Sayre Syndrome and the mitochondrial encephalopathy, lactic-acidosis and stroke-like episodes (MELAS) syndrome [1]. More recently, mtDNA defects have also been found

**Key words:** differential display, ethidium bromide, mitochondrial DNA, mRNA, toxicity.

**Abbreviations:** ARCN, archain vesicle transport protein; DDRT-PCR, differential display reverse transcriptase-PCR; EtBr, ethidium bromide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MELAS syndrome, mitochondrial encephalopathy, lactic-acidosis and stroke-like episodes syndrome; mtDNA, mitochondrial DNA;  $\rho^+$ ,  $\rho^0$ , mtDNA-replete and mtDNA depleted cells respectively.

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in pedigrees with diabetes and deafness [2–4]. Although there is little doubt that certain mtDNA defects are pathogenic, the precise mechanism by which they cause disease is unclear. A number of issues relating to mitochondrial disorders remain poorly understood. First, the same mtDNA mutation may be associated with a wide range of clinical phenotypes. Thus the A-to-G substitution at position 3243 in the mitochondrial tRNA<sup>Leu(UUR)</sup> gene has been reported in patients with life-threatening MELAS syndrome [5], diabetes and deafness (but no other neurological deficit) and gestational diabetes, and in apparently healthy maternal relatives of affected individuals [2,3]. It has been suggested that these differences are a manifestation of heteroplasmy and segregative replication, by which different cells and tissues in the body have different proportions of mutated mtDNA [6]. Although this is a plausible explanation, the muscle of some patients with only diabetes and nerve deafness has been shown to harbour the 3243 mutation at levels similar to those reported in patients with clinically apparent myopathy [7]. It should be noted, however, that this does not preclude differences in the overall quantity of mtDNA between individuals or differential segregation of wild-type and mutant mtDNA as an explanation for the disparate phenotypes. Nevertheless, the same clinical phenotype can result from a variety of different mtDNA defects at different positions within the genome. Thus Leber's hereditary optic neuropathy has been associated with at least six primary mtDNA defects in widely separated regions of the mitochondrial genome [8,9]. One hypothesis is that mtDNA defects cause impaired oxidative phosphorylation and, once intracellular ATP levels fall below a particular threshold, disease results. The different phenotypes are seen as a manifestation of the variable heteroplasmy and ATP requirements in tissues of different individuals harbouring mtDNA mutations.

An alternative hypothesis is that the disease phenotype results from the altered expression of nuclear genes, driven either by ATP deficiency or by the influence of mutated mtDNA. Although the mitochondrial genome may be considered to be isolated from the nucleus, a great deal of communication between the nucleus and mitochondria occurs. The nuclear genes encode all the factors responsible for controlling mtDNA replication and transcription [10–13]. Diseases associated with mtDNA depletion [14,15] and multiple mtDNA deletions [16–18] are autosomally inherited. This suggests that nuclear gene defects may be manifested as mitochondrial disorders, and confirms the importance of the nuclear genome in mtDNA physiology. Likewise, mtDNA may affect nuclear DNA expression. In yeast, the quality and quantity of mtDNA has been shown to modulate the levels of nuclear-encoded RNAs [19] and, in cultured chicken cells, subtractive hybridization techniques have

recently shown that inhibition of mtDNA expression is associated with the up-regulation of a number of nuclear genes [20].

In recent years the technique of depleting cells of mtDNA has been employed to investigate the mechanisms underlying mitochondrial disorders [21]. It was found that the addition of ethidium bromide (EtBr) to culture media resulted in a progressive depletion of intracellular mtDNA levels, leading eventually to complete and permanent loss of mtDNA. Such cells (termed  $\rho^0$  cells) remain viable in culture due to anaerobic metabolism, but are auxotrophic for uridine. Although a widely used technique, the precise mechanism by which EtBr results in the  $\rho^0$  state is unknown, but it is accepted that the compound has a wide variety of other effects on cells, including the promotion of nuclear gene defects.

The aim of the present study was to investigate the effects of EtBr exposure in a human thyroid cell line. The molecular differences in gene expression between normal (wild-type) cells ( $\rho^+$ ) and mtDNA-depleted cells ( $\rho^0$ ) were studied using the techniques of mRNA differential display reverse transcriptase-PCR (DDRT-PCR) [22] and Northern blot analysis.

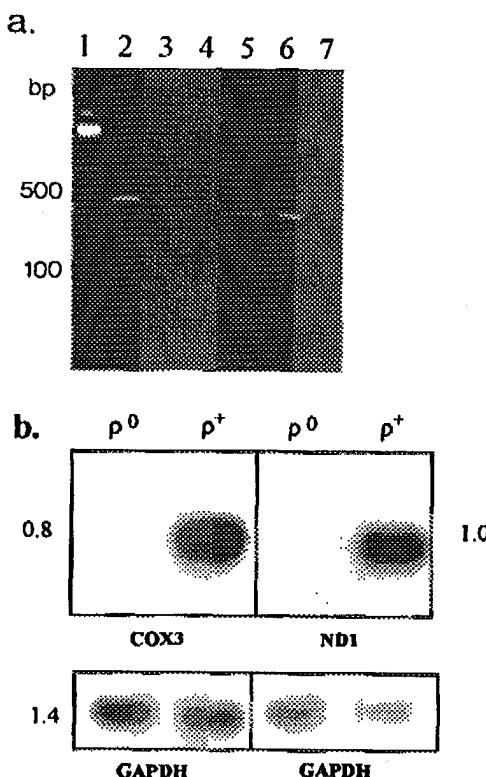
## METHODS

### Cells

Cells may be depleted of mtDNA by co-culture with EtBr. This technique has been used previously to produce human fibroblast and myoblast cell lines totally devoid of mtDNA ( $\rho^0$ ) [21]. Such  $\rho^0$  cells remain viable by anaerobic glycolysis provided that excesses of glucose, uridine and pyruvate are added to the culture medium. Since mtDNA disorders have been associated with endocrine defects, we chose to manipulate a human thyroid cell line. Briefly, a simian-virus-40-transformed human thyroid cell line (Ori3) [23] was maintained in RPMI 1640 medium supplemented with 1 mM pyruvate, 50 µg/ml uridine, 25 mg/ml glucose and 10% (v/v) fetal calf serum. A single colony of cells was allowed to become confluent in a cell culture flask and then was divided into two separate 10 cm-diam. culture dishes. EtBr (50 nM) was added to the culture medium of one of the dishes. The cells were then maintained in culture for a total of approx. 30 cell divisions with successive passage into either normal ( $\rho^+$  cells) or EtBr-supplemented ( $\rho^0$  cells) medium. Prior to mRNA extraction, EtBr was excluded from the medium of  $\rho^0$  cells, so that all cells had been growing in identical media for at least four passages.

### mRNA preparation

A single 10 cm-diam. culture dish of confluent cells [approx.  $(2\text{--}4) \times 10^6$  cells] was used for mRNA isolation. An acid guanidinium thiocyanate/phenol/chloroform RNA extraction kit was used (Ultraspec; Biotez Labs,



**Figure 1.** Amplification of mitochondrial and nuclear gene sequences in  $\rho^0$  and  $\rho^+$  cells (a) and Northern blot analysis of mitochondrially encoded subunits of oxidative phosphorylation in  $\rho^0$  and  $\rho^+$  cells (b)

(a) Lane 1, 100 bp molecular size ladder; lanes 2–4, mitochondrial tRNA<sup>Leu(UUR)</sup> gene (428 bp) for  $\rho^+$ ,  $\rho^0$  and negative control cells respectively; lanes 5–7, nuclear ApoCIII gene (400 bp) for  $\rho^+$ ,  $\rho^0$  and negative control respectively. (b) COX3, cytochrome oxidase 3; NDI, NADH dehydrogenase complex 1. After stripping the membranes, GAPDH was used as the control nuclear gene probe to confirm equal RNA loading. Molecular sizes of RNAs are shown in kb.

Witney, Oxford, U.K.). mRNA was routinely treated with DNase I to remove any DNA template.

#### DDRT-PCR technique

Reverse transcription of RNA was carried out essentially as described by Liang and Pardee [22]. Four individual anchor primers were used to generate cDNA: dT<sub>12</sub>AG, dT<sub>12</sub>AC, dT<sub>12</sub>CT and dT<sub>12</sub>GC. Three 10-mer arbitrary primers were used in conjunction with each anchored primer for amplification of cDNA. The arbitrary primer sequences were as follows; Arb 1, CTTGATTGCC; Arb 2, GAATACGCCG; Arb 3, GATCTCAGAC.

PCR was carried out in a total volume of 20  $\mu$ l, which contained 2.5  $\mu$ l of dT<sub>12</sub>MN, 0.5  $\mu$ M of the respective arbitrary upstream primer, 2  $\mu$ M of each dNTP, 2  $\mu$ Ci (60 nM) of [<sup>32</sup>P]dATP, 10 mM Tris/HCl (pH 8.3),

50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 1 unit of *Taq* polymerase. A total of 40 amplification cycles were performed at 94 °C for 30 s, 40 °C for 2 min and 72 °C for 30 s, followed by an extension period at 72 °C for 5 min, using an Omniprime thermocycler (Hybaid, Teddington, U.K.). Each PCR reaction was carried out in triplicate.

#### Gel electrophoresis

PCR samples were mixed with an equal volume of 90% (v/v) formamide dye solution and heated to 80 °C for 2 min, and a 3.5  $\mu$ l aliquot was run on a 6% (w/v) polyacrylamide/urea gel in Tris-borate buffer for approx. 3.5 h at 60 W (potential difference < 1700 V). The gel was then transferred to 3M Whatman paper, dried on a gel dryer (Appligene Oncor, Durham, U.K.) and exposed to X-ray film overnight at -70 °C.

#### Amplification of DNA fragments

Differentially displayed fragments were eluted from the dried gel, ethanol-precipitated and re-amplified in a reaction volume of 40  $\mu$ l using the same PCR conditions as described above, except that the dNTP concentrations were increased to 20  $\mu$ M, the concentration of the arbitrary 10-mer primer was reduced to 0.2  $\mu$ M and the isotope was omitted.

#### Cloning and screening of DNA fragments

Re-amplified PCR products were cloned using a TA overhang vector system (LigATor; R&D Systems, Abingdon, Oxon., U.K.). Briefly, PCR products generated from eluted cDNAs were ligated into the pTAg plasmid and transformed into competent cells (supplied by R&D Systems). Clonality of cDNAs was ensured by isolation of plasmid DNA from individual recombinant clones. Specific DNA fragments were then PCR-amplified, purified and sequenced using an automated *Taq* polymerase reaction. Sequence data were analysed using the FASTA program to screen the EMBL, GenBank and EST (expressed sequence tag) libraries via the MRC HGMP Research Centre (Cambridge, U.K.). Once characterized, cloned cDNAs were used as probes in Northern blots to confirm the differential expression of their respective mRNAs.

#### Northern blot analysis

Samples of total RNA (10  $\mu$ l each) were heated to 65 °C prior to seeding in lanes of a 1% (w/v) agarose/formaldehyde gel, followed by electrophoresis in 1% MOPS buffer at 60 V for approx. 2 h.

Labelling of PCR product probes was performed using the Prime-a-Gene labelling kit (Promega) incorporating [<sup>32</sup>P]dATP. Hybridization was carried out using 25 ng of heat-denatured labelled probe per 10 ml of hybridization

buffer comprising  $6 \times$  SSC,  $5 \times$  Denhardt's solution,  $100 \mu\text{g}/\text{ml}$  denatured salmon sperm DNA and  $0.5\%$  SDS ( $1 \times$  SSC is  $0.15\text{ M NaCl}/0.015\text{ M sodium citrate}$ , and  $1 \times$  Denhardt's is  $0.02\%$  Ficoll 400/ $0.02\%$  polyvinylpyrrolidone/ $0.002\%$  BSA). Membranes were incubated for approx.  $16\text{ h}$  at  $60^\circ\text{C}$ . The blots were then washed twice in  $1 \times$  SSC/ $0.1\%$  SDS at room temperature for  $15\text{ min}$ , followed by two washes with  $0.25 \times$  SSC/ $0.1\%$  SDS at  $55^\circ\text{C}$  for  $30\text{ min}$  prior to exposure to X-ray film.

In addition to the PCR product probes generated from the DDRT-PCR procedures, Northern analysis was also performed using probes for mitochondrial transcription factor 1 (mtTFA; donated by Professor D. Clayton, Howard Hughes Medical Institute, MD, U.S.A.), cytochrome oxidase VII (pCox 7.22; donated by Dr. E. Schon, Columbia University, New York, NY, U.S.A.) and thyroglobulin [23]. Control probes [either  $\beta$ -actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)] were used on all blots to confirm equal RNA loading.

## RESULTS

### mtDNA depletion

Ori3 cells grew well in media supplemented with EtBr. After  $30\text{ cell divisions}$ , removal of uridine from the culture medium resulted in cell death; uridine dependence is a well-recognized feature of  $\rho^0$  cell lines. Uridine dependence was observed even several months after  $\rho^0$  cells were removed from EtBr exposure, suggesting that the mtDNA depletion was permanent. Lack of mtDNA was confirmed by an inability to generate PCR products of specific mitochondrial genes using template DNA extracted from the EtBr-treated cells (Figure 1a) and the absence of detectable mtDNA gene products in Northern

blot analysis of RNA extracted from these cells (Figure 1b).

### Differential display gels

Three arbitrary 10-mer primers were screened against four  $\text{T}_{12}\text{MN}$  primers. Each differential display lane yielded between  $100$  and  $200$  discrete bands. We identified seven clones which were down-regulated in  $\rho^0$  cells (Table 1 and Figure 2). The differential expression of five of these clones was confirmed by Northern blot analysis (Figure 3), but the other two did not produce detectable bands in either  $\rho^+$  or  $\rho^0$  cell lines. We also identified two unknown cDNAs on differential display gels that appeared to show increased expression in  $\rho^0$  cells (Table 1 and Figure 2). However, the differential expression of these clones could not be substantiated by subsequent Northern blot analysis.

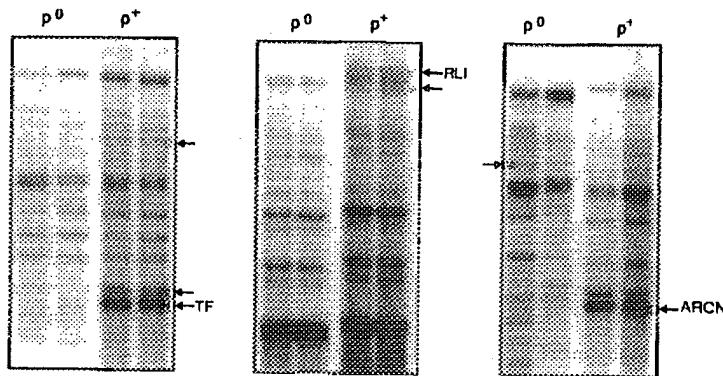
Control probes used in Northern blot analysis included  $\beta$ -actin, GAPDH and clone numbers 008/7 and 011/1. Clones 008/7 and 011/1 were generated from fragments which showed equal expression on differential display gels in both  $\rho^+$  and  $\rho^0$  cell lines. Sequencing of these clones showed that they had  $> 80\%$  identity with human ribosomal protein S5 mRNA and a human housekeeping gene (Q1Z7F5) respectively (Table 1). All control probes showed equal expression in both  $\rho^+$  and  $\rho^0$  cell lines (Figure 3).

Of the seven differentially expressed clones that showed reduced expression in  $\rho^0$  cells, three were similar to known human gene sequences [RNase L inhibitor, ARCN1 (archain vesicle transport protein 1) and human tissue factor] and the four others matched with previously sequenced cDNAs from currently unknown genes (Table 1). The two clones that demonstrated increased ex-

**Table 1** cDNA clones isolated using DDRT-PCR analysis of  $\rho^0$  and  $\rho^+$  thyroid cells

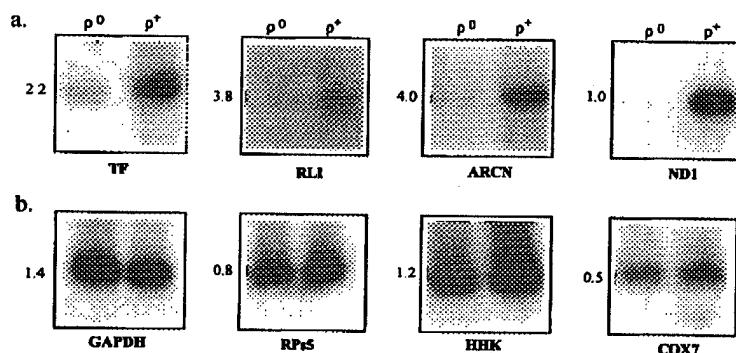
Key to expression in  $\rho^0$  cells: —, reduced expression; +, increased expression; =, equal expression in  $\rho^0$  and  $\rho^+$  cells. 'Identity (%)' gives the percentage identity of the cloned fragment with the indicated DNA sequences in the Genbank (GB) or EMBL (EM) databases (only the best match is shown). The length of sequence overlap is given in parentheses. EST, expressed sequence tag.

Clone no.	Primers	Expression in $\rho^0$ cells	Size of fragment (bp)	Identity (%)
001/16	pT <sub>12</sub> AG/Arb 2	—	608	95% (428 nt); RNase L inhibitor (2'-5A binding protein), 2861 nt; GB: HSBINDPR X74987
003/15	pT <sub>12</sub> CA/Arb 3	—	552	77% (452 nt); EM: HS1273015 AA480855 aa282a2.s1; 457 nt cDNA clone
003/16	pT <sub>12</sub> CA/Arb 3	—	555	70% (335 nt); EM: HS49J10 Z84572; 74413 nt cDNA clone
003/20	pT <sub>12</sub> CA/Arb 3	—	550	73% (467 nt); GB: W63681 zd30d01.s1; 608 nt cDNA clone
004/2	pT <sub>12</sub> CA/Arb 3	—	414	80% (339 nt); human tissue factor, I3865 nt; EM: HSTFPB J02846
005/1	pT <sub>12</sub> CA/Arb 1	—	410	77% (371 nt); EM: HSXT00848 M78700; 378 nt EST
005/3	pT <sub>12</sub> CA/Arb 1	—	430	76% (337 nt); ARCN, 3701 nt; GB: HSARCPS X81198
006/3	pT <sub>12</sub> CA/Arb 1	+	550	95% (184 nt); EM: HSAA36216 AAI36216 zn89b04.s1; 465 nt cDNA clone
007/3	pT <sub>12</sub> CA/Arb 3	+	220	78% (213 nt); GB: AAI3681 z102a05.s1; 475 nt cDNA clone
008/7	pT <sub>12</sub> AG/Arb 2	=	384	80% (228 nt); human ribosomal protein S5, 705 nt; GB: HSU14970 U14970
011/1	pT <sub>12</sub> AG/Arb 2	=	310	97% (171 nt); human housekeeping gene (Q1Z7F5), 3188 nt; GB: HUMHSKPQZ M81806



**Figure 2** Three separate examples of differential display gels showing the presence of differentially expressed cDNA fragments in  $p^0$  and  $p^+$  cells

Sample lanes are shown in duplicate. Signals showing altered expression are marked by arrows. Fragments with known identity are indicated as follows: TF, human tissue factor; RLI, RNase L inhibitor; ARCN, Archain vesicle transport protein.



**Figure 3** Northern blot analysis of  $p^0$  and  $p^+$  cells

(a) Differentially expressed cDNAs. From left to right: human tissue factor (TF), RNase L inhibitor (RLI), ARCN and NADH dehydrogenase complex I (ND1). (b) Non-differentially expressed cDNAs. From left to right: GAPDH, ribosomal protein s5 (Rps5), human housekeeping gene (HHK) and nuclear-encoded cytochrome oxidase 7 (COX7). Molecular sizes of RNAs (in kb) are shown on the left of each panel.

pression in  $p^0$  cells showed > 80% identity with unidentified cDNA clones. However, as noted above, we were unable to confirm this increased expression by Northern blot analysis.

Northern blot analysis confirmed the absence of mitochondrial encoded cytochrome oxidase 3 and NADH dehydrogenase complex I subunits from the  $p^0$  cells (Figure 1b). None of the differentially expressed clones isolated from the DDRT-PCR gels represented a mitochondrial gene. This is not surprising, since the mitochondrial genome only encodes 13 enzyme subunits, whereas > 15000 mRNA species are thought to arise from the nucleus. Thus the technique will be insensitive to the small number of mitochondrial mRNA sequences compared with the large number of cDNAs of nuclear origin. Northern blot analysis of the nuclear-encoded

subunit of the mitochondrial cytochrome oxidase 7 enzyme and mitochondrial transcription factor 1 showed equal expression in both  $p^0$  and  $p^+$  cell lines (Figures 1b and 3). No thyroglobulin mRNA could be detected in either cell type.

## DISCUSSION

The supplementation of culture medium with EtBr resulted in the production of a human thyroid cell line that was depleted of mtDNA. Unfortunately, as is common with human endocrine cell lines, de-differentiation took place during the repeated passages required for these experiments, and thyroglobulin mRNA

could not be detected in these cells. The technique of DDRT-PCR proved a robust and reproducible method to compare mRNA expression between wild-type and EtBr-treated cell lines. With the primers used in this study we identified seven clones which showed decreased expression in the cells cultured with EtBr. Two clones appeared to show overexpression in the treated cells, but it was not possible to confirm this finding on repeated Northern analysis. A number of conclusions may be reached from these findings. First, the vast majority of mRNAs within a cell are quantitatively unaffected by EtBr treatment (and consequent mtDNA depletion). These include those for the nuclear-encoded subunits of the mitochondrial oxidative phosphorylation pathway, such as cytochrome oxidase 7. Although initially surprising, such a finding may indicate that these nuclear genes are activated constitutively and that expression occurs irrespective of mtDNA or ATP levels within the cell. Secondly, although we did not use all the possible primer combinations in this study, the total number of differentially expressed genes is likely to be fairly small, and amenable to further investigation by currently available techniques. As a corollary to this, concerns about the validity of EtBr-generated  $\rho^0$  cell lines in the study of mitochondrial disorders caused by the possible generation of widespread 'bystander' nuclear gene defects seem to be at least partly alleviated. Thirdly, although we were able to positively match the sequences of three differentially expressed clones to known genes, the majority represented sequences deposited in cDNA and EST (expressed sequence tag) libraries, for which the full gene and function are unknown. As the Human Genome Mapping Project develops, these 'anonymous' mRNAs should become more clearly defined, but currently the only approach to clarifying the results of DDRT-PCR would be to attempt sequencing and characterization of the full-length cDNAs derived from the differentially expressed clones.

It is important to be aware of the limitations of this technique. In the present study, reproducible results could only be obtained if differential expression was defined as a clear and unequivocal difference between adjacent lanes of a gel. This means that we are likely to have found only those mRNAs for which there is a large difference in expression (e.g. those almost totally switched off by mtDNA depletion). It is not possible to exclude subtle changes in other genes, and it would be simplistic to assume that only large changes in mRNA levels are of pathophysiological significance.

Despite these limitations, we identified three clones that were consistently underexpressed in Ori3 cells exposed to EtBr during long-term culture, and the sequences of these clones showed similarity to those of RNase L inhibitor, a vesicle transport protein (ARCNI) and human tissue factor. These results were consistent when RNA was extracted from a number of Ori3 cell

populations that had been treated with EtBr on separate occasions. Further studies are required to confirm whether these genes are similarly affected in non-Ori3 cell lines.

RNase L inhibitor is a factor in the interferon-regulated 2-5A system thought to play a key role in the control of RNA stability [24]. It may also play a part in transducing interferon function. The expression of RNase L inhibitor has been shown to be tightly correlated with cellular growth rates and the state of cell differentiation [25]. Furthermore, the activity of the 2-5A system is dependent upon ATP levels [26]. Thus the ATP deficiency within EtBr-treated cells might explain the down-regulation of RNase L inhibitor. While it could be postulated that RNase L inhibitor deficiency in  $\rho^0$  cells may contribute to generalized RNA instability, and that this might play a role in disease development, there is currently no direct evidence to support this contention. Similarly, the reduced expression of human tissue factor in  $\rho^0$  cells is difficult to link directly to the phenotypes seen in the various human mitochondrial disorders. We are unaware of any published reports of the levels of human tissue factor or other components of the coagulation pathway in patients with stroke-like episodes associated with MELAS syndrome.

The finding of decreased expression of an ARCN1-like sequence, which is related to proteins involved in vesicle structure and trafficking [27], is of interest, given the frequent occurrence of endocrine disturbance in patients with mitochondrial disorders. In particular, diabetes associated with mtDNA defects is characterized by impaired glucose-stimulated insulin secretion. Studies in cybrid cells have suggested normal levels of insulin mRNA in mtDNA-depleted rodent islet cells with a defect of vesicle-mediated insulin release [28]. The presence of ARCN1 mRNA has been detected in pancreatic tissue in association with insulin secretory vesicles [27]. Therefore the possibility exists that decreased expression of this or another vesicle transport protein may underlie the endocrine disturbance seen in some patients with mitochondrial diseases.

In summary, DDRT-PCR of wild-type and EtBr-treated Ori3 cells reveals that most nuclear genes, including those coding for subunits of the oxidative phosphorylation pathway, show no obvious change in expression on EtBr treatment. Some genes are differentially expressed and, although it is possible to speculate on possible links with mtDNA depletion and the mitochondrial disorders, further studies will be required to elucidate whether the changes are due to mtDNA depletion, ATP deficiency, or perhaps direct effects of EtBr on nuclear genes that are entirely independent of the effects on mtDNA and ATP production. The EtBr-induced  $\rho^0$  model will continue to be valuable in isolation as a model of mitochondrial disease and also for the study of pathogenic mtDNA mutations.

## ACKNOWLEDGMENTS

This work was funded by the British Diabetic Association and Welsh Scheme for Health and Social Research. We are grateful to Dr. M. P. King for hosting a visit to Columbia University, where J. C. A. learnt the techniques for mtDNA depletion. The visit was funded by the Leonard Simpson Travelling Fellowship of the Royal College of Physicians, London, U.K. M. P. King, E. Schon and D. Clayton kindly provided some of the mRNA probes, and the Ori3 cells were provided by Professor D. Wynford-Thomas, University of Cardiff.

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Received 30 November 1998/17 February 1999; accepted 8 April 1999